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**WO 01/08710 A1**

(54) Title: GENE EXPRESSION WITH COVALENTLY MODIFIED POLYNUCLEOTIDES

(57) Abstract: A process and compound wherein nucleic acids can be modified with a host of molecules and maintain their ability to be expressed. A modifying chemical attachment of polyions to polynucleotides can be used to facilitate the change of tertiary structure of the nucleic acid and in some cases condensation of nucleic acids into smaller, charged particles useful in delivering the nucleic acid to a cell.

## Gene Expression With Covalently Modified Polynucleotides

This application is related to prior provisional application 60/146,824 filed on August 2, 1999.

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### Field

This invention relates to the use of covalently modified polynucleotides for use in gene delivery and gene therapy applications. More specifically, polynucleotides can be modified, using a modifying chemical attachment bonding method of attachment, with a host of molecules and maintain their ability to be expressed. The modifying chemical attachment attachment, in some cases a covalent attachment, of polyions to polynucleotides can be used to facilitate the change of tertiary structure of the nucleic acid and in some cases condensation of nucleic acids into smaller, charged particles useful in delivery.

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### Background

#### *Nucleic Acid Modification*

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Polynucleotides can be covalently modified using a large number of different methodologies. Direct covalent attachment of molecules to polynucleotides can be accomplished by nitrogen mustards (alkylation), 2-acetylfluorene, 4-aminohydroxylamine, p-diazobenzoyl-biotin, bisulfite activation, n-bromosuccinimide activation, EDC modification of 5' phosphates and with photobiotin (N-(4-azido-2-nitrophenyl)-aminopropyl-N'-(N-d-biotinyl-3-aminopropyl)-N'-methyl-1,3-propanediamine. Polynucleotides (DNA or RNA) can also be synthesized via *in vitro* enzymatic reactions to include covalently modified nucleotides. These modified nucleotides, which are incorporated into the growing polynucleotide chain, can be chemically coupled to a wide array of molecules. Some examples of molecules that can be covalently linked to polynucleotides directly or through enzymatic incorporation of modified nucleotides include; fluorescent molecules (fluorescein, rhodamine, Cy-2, Cy-3, Cy-5), peptides (i.e. nuclear

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localizing signals), proteins (enzymes, ligands, antibodies), lipids, sugars, carbohydrates, biotin, avidin, streptavidin, chemiluminescent substrates, digoxin and dinitrophenyl (DNP). Thus using any of these compounds or methods a vast array of molecules or compounds can be covalently attached to polynucleotides.

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### ***Nucleic Acid Alkylation***

Nucleic acid alkylation results in the formation of a chemical bond between the alkylating compound (labeling reagent) and the polynucleic acid. In an alkylation  
10 reaction the polynucleic acid is incubated with the said compounds in aqueous or non-aqueous solutions, followed by separation of the labeled polynucleic acid from the unreacted alkylating reagent. The extent of alkylation can be controlled by regulating the relative amounts of alkylating reagent and polynucleic acid, by adjusting the length of the incubation, by controlling the temperature of the incubation, by  
15 controlling the absolute concentrations of polynucleic acid and alkylating reagent, and by controlling the composition of the aqueous or organic solution using solvent, pH, ionic strength, and buffers.

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### ***Gene Therapy***

Delivery of functional polynucleotides or other genetic material for therapeutic purposes is gene therapy. Commercial uses of delivery and transfection processes  
25 include the purchase of such a process by a corporation or other institution for use in systems which incorporate a delivery process whether the use is for further preparation for future commercial sale or direct sale.

A polynucleotide can be delivered to a cell in order to produce a cellular change that  
30 is therapeutic. Polynucleotides may be coded to express a whole or partial protein, or may be anti-sense, and can be delivered either directly to the organism in situ or indirectly by transfer to a cell that is then transplanted into the organism. The protein

can be missing or defective in an organism as a result of a genetic, inherited or acquired defect in its genome.

For example, a polynucleotide may be coded to express the protein dystrophin that is missing or defective in Duchenne muscular dystrophy. The coded polynucleotide is delivered to a selected group or groups of cells and incorporated into those cell's genome or remain apart from the cell's genome. Subsequently, dystrophin is produced by the formerly deficient cells. Other examples of imperfect protein production that can be treated with gene therapy include the addition of the protein clotting factors that are missing in the hemophilias and enzymes that are defective in inborn errors of metabolism such as phenylketonuria (PKU).

A delivered polynucleotide can also be therapeutic in acquired disorders such as neurodegenerative disorders, cancer, heart disease, and infections. The polynucleotide has its therapeutic effect by entering the cell. Entry into the cell is required for the polynucleotide to produce the therapeutic protein, to block the production of a protein, or to decrease the amount of a RNA. Other therapeutic genes can be erythropoietin, vascular growth factors such as fibroblast growth factor (FGF) or vascular endothelial growth factor (VEGF).

A delivered polynucleotide can also be therapeutic by stimulating or inducing a strong immune response against a desired foreign antigen. The polynucleotide has its therapeutic effect by entering the cell and being expressed into protein. This protein, upon being recognized as "foreign" by the immune system stimulates the production of both antibody and/or cell mediated protective responses against the expressed protein.

Additionally, a polynucleotide can be delivered to block gene expression. Such polynucleotides can be anti-sense by preventing translation of a messenger RNA or could block gene expression by preventing transcription of the gene. Preventing RNA translation and/or DNA transcription is considered preventing expression. Transcription can be blocked by the polynucleotide binding to the gene as a duplex or

triplex. It could also block expression by binding to proteins that are involved in a particular cellular biochemical process.

Polynucleotides may be delivered that recombine with genes. The polynucleotides  
5 may be DNA, RNA, hybrids and derivatives of natural nucleotides. Recombine is the mixing of the sequence of a delivered polynucleotide and the genetic code of a gene. Recombine includes changing the sequence of a gene.

Delivery of a polynucleotide means to transfer a polynucleotide from a container  
10 outside a mammal to within the outer cell membrane of a cell in the mammal. The term transfection is used herein, in general, as a substitute for the term delivery, or, more specifically, the transfer of a polynucleotide from directly outside a cell membrane to within the cell membrane. If the polynucleotide is a messenger RNA, a ribosome translates the messenger RNA to produce a protein within the cytoplasm. If  
15 the polynucleotide is a DNA, it enters the nucleus where it is transcribed into a messenger RNA that is transported into the cytoplasm where it is translated into a protein. The polynucleotide contains sequences that are required for its transcription and translation. These include promoter and enhancer sequences that are required for initiation. DNA and thus the corresponding mRNA (transcribed from the DNA)  
20 contains introns that must be spliced, poly A addition sequences, and sequences required for the initiation and termination of its translation into protein. Therefore if a polynucleotide expresses its cognate protein, then it must have entered a cell.

A therapeutic effect of the protein in attenuating or preventing the disease state can be  
25 accomplished by the protein either staying within the cell, remaining attached to the cell in the membrane or being secreted and dissociating from the cell where it can enter the general circulation and blood. Secreted proteins that can be therapeutic include hormones, cytokines, growth factors, clotting factors, anti-protease proteins (e.g. alpha-antitrypsin) and other proteins that are present in the blood. Proteins on the  
30 membrane can have a therapeutic effect by providing a receptor for the cell to take up a protein or lipoprotein. For example, the low density lipoprotein (LDL) receptor could be expressed in hepatocytes and lower blood cholesterol levels by removing excess LDL from the blood and thereby prevent atherosclerotic lesions that can cause

strokes or myocardial infarction. Therapeutic proteins that stay within the cell can be enzymes that clear a circulating toxic metabolite as in phenylketonuria. They can also cause a cancer cell to be less proliferative or cancerous (e.g. less metastatic). A protein within a cell could also interfere with the replication of a virus.

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The terms "therapeutic" and "therapeutic results" are defined in this application as levels of gene products, including reporter (marker) gene products, which indicate a reasonable expectation of gene expression using similar compounds (nucleic acids), at levels considered sufficient by a person having ordinary skill in the art of gene

10 therapy. For example: Hemophilia A and B are caused by deficiencies of the X-linked clotting factors VIII and IX, respectively. Their clinical course is greatly influenced by the percentage of normal serum levels of factor VIII or IX: < 2%, severe; 2-5%, moderate; and 5-30% mild. This indicates that in severe patients only 2% of the normal level can be considered therapeutic. Levels greater than 6% prevent

15 spontaneous bleeds but not those secondary to surgery or injury. A person having ordinary skill in the art of gene therapy would reasonably anticipate therapeutic levels of expression of a gene specific for a disease based upon sufficient levels of marker gene results. In the Hemophilia example, if marker genes were expressed to yield a protein at a level comparable in volume to 2% of the normal level of factor VIII, it

20 can be reasonably expected that the gene coding for factor VIII would also be expressed at similar levels.

#### **Nucleic acids (Polynucleotides)**

25 The term nucleic acid is a term of art that refers to a string of at least two base-sugar-phosphate combinations. (A polynucleotide is distinguished, here, from an oligonucleotide by containing more than 120 monomeric units.) The term includes deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). Nucleotides are the monomeric units of nucleic acid polymers. Anti-sense is a polynucleotide that

30 interferes with the function of DNA and/or RNA. Natural polynucleotides have a phosphate backbone, artificial polynucleotides may contain other types of backbones, but contain the same bases. RNA may be in the form of an tRNA (transfer RNA), snRNA (small nuclear RNA), rRNA (ribosomal RNA), mRNA (messenger RNA),

anti-sense RNA, and ribozymes. DNA may be in form plasmid DNA, viral DNA, linear DNA, or chromosomal DNA or derivatives of these groups. In addition to these forms; DNA and RNA may be single, double, triple, or quadruple stranded. The term also includes PNAs (peptide nucleic acids), phosphorothioates, and other  
5 variants of the phosphate backbone of native nucleic acids.

A delivered DNA can stay within the cytoplasm or nucleus apart from the endogenous genetic material. Alternatively, the DNA could recombine (become a part of) the endogenous genetic material. For example, DNA can insert into chromosomal DNA  
10 by either homologous or non-homologous recombination.

### ***Condensation of DNA***

A large number of multivalent cations with widely different molecular structures have  
15 been shown to induce change in the tertiary structure of DNA including condensation.

Two approaches are currently used for compacting (condensing) DNA:

1. Multivalent cations with a charge of three or higher have been shown to condense  
20 DNA. These include spermidine, spermine,  $\text{Co}(\text{NH}_3)_6^{3+}$ ,  $\text{Fe}^{3+}$ , and natural or synthetic polymers such as histone H1, protamine, polylysine, and polyethylenimine. Analysis has shown DNA condensation to be favored when 90% or more of the charges along the sugar-phosphate backbone are neutralized by the polycation.
2. Polymers (neutral or anionic) which can increase repulsion between DNA and its  
25 surroundings have been shown to compact DNA. Most significantly, spontaneous DNA self-assembly and aggregation process have been shown to result from the confinement of large amounts of DNA, due to excluded volume effect.

Depending upon the concentration of DNA, condensation leads to three main types of  
30 structures:

- 1) In extremely dilute solution (about 1  $\mu\text{g}/\text{ml}$  or below), long DNA molecules can undergo a monomolecular collapse and form structures described as toroids.

2) In very dilute solution (about 10 ug/ml) microaggregates form with short or long molecules and remain in suspension. Toroids, rods and small aggregates can be seen in such solution.

3) In dilute solution (about 1 mg/ml) large aggregates are formed that sediment readily.

5

Toroids have been considered an attractive form for gene delivery because they have the smallest size. While the size of DNA toroids produced within single preparations has been shown to vary considerably, toroid size is unaffected by the length of DNA being condensed. DNA molecules from 400 bp to genomic length produce toroids comparable in size. Therefore one toroid can include from one to several DNA molecules. The kinetics of DNA collapse by polycations that resulted in toroids is very slow. For example DNA condensation by  $\text{Co}(\text{NH}_3)_6\text{Cl}_3$  needs 2 hours at room temperature.

15

The mechanism of DNA condensation is not obvious. The electrostatic force between unperturbed helices arises primarily from a counterion fluctuation mechanism requiring multivalent cations and plays a major role in DNA condensation. The hydration forces predominate over electrostatic forces when the DNA helices approach closer than a few water diameters. In the case of DNA - polymeric polycation interactions, DNA condensation is a more complicated process than the case of low molecular weight polycations. Different polycationic proteins can generate toroid and rod formation with different size DNA at a ratio of positive to negative charge of 0.4. T4 DNA complexes with polyarginine or histone can form two types of structures; an elongated structure with a long axis length of about 350 nm (like free DNA) and dense spherical particles. Both forms exist simultaneously in the same solution. The reason for the co-existence of the two forms can be explained as an uneven distribution of the polycation chains among the DNA molecules. The uneven distribution generates two thermodynamically favorable conformations. The electrophoretic mobility of DNA-polycation complexes changes from negative to positive when there is an excess of polycation. This results in DNA condensation and particle formation and the DNA-polycation complexes remain in the well during electrophoresis. In the absence of an excess of a polycation or oligocation, the DNA

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remains unaggregated and the DNA and polycations can dissociate allowing the DNA to migrate into an agarose gel during electrophoresis. In DNA/polycation complexes (not covalently or chemically attached) it is likely that the large polycations don't completely align along DNA but form polymer loops that interact with other DNA molecules. The rapid aggregation and strong intermolecular forces between different DNA molecules may prevent the slow adjustment between helices needed to form tightly packed orderly particles.

### ***Transfection Reagents***

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A transfection reagent is a compound or compounds used in the prior art that bind(s) to or complex(es) with polynucleotides and mediates their entry into cells. The transfection reagent also mediates the binding and internalization of polynucleotides into cells. Examples of transfection reagents include cationic liposomes and lipids, amphipathic polyamines, polyethylenimine, calcium phosphate precipitates, and polylysine complexes. Typically, the transfection reagent has a net positive charge that binds to the polynucleotide's negative charge. The transfection reagent mediates binding of polynucleotides to cells via its positive charge (that binds to the cell membrane's negative charge) or via ligands that bind to receptors in the cell. For example, cationic liposomes or polylysine or polyethylenimine (PEI) complexes have net positive charges that enable them to bind to DNA. Other vehicles are also used, in the prior art, to transfer genes into cells. These include complexing the polynucleotides on particles that are then accelerated into the cell. This is termed biolistic or gun techniques. Other methods include electroporation in which a device is used to provide a electric current or charge to cells.

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### ***Naked DNA***

Naked DNA can also be used for gene therapy. The term, naked DNA or polynucleotides, indicates that the polynucleotides are not associated with a transfection reagent or other delivery vehicle that is required for the polynucleotide to be delivered to the parenchymal cell. The naked DNA can be delivered by direct intraparenchymal injection into the tissue or can be delivered by an intravascular

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route. These tissues can include striated muscle (e.g. heart and skeletal muscle), liver, lung, intestines, brain, adrenal glands, thymus, kidneys, brain, spinal cord, peripheral nerves, endothelial cells, blood vessels, spleen, gonads, thyroid, skin, pancreas, salivary glands, eyes, mucosal membranes, vagina, bladder, prostate, cancer cells,  
5 tumors, neoplastic tissue, and blood cells (e.g. leukocytes, platelets, red blood cells).

### ***Vectors***

Vectors are polynucleic acid molecules originating from a virus, a plasmid, or the cell  
10 of a higher organism into which another nucleic fragment of appropriate size can be integrated; vectors introduce foreign DNA into host cells, where it can be reproduced. Examples are plasmids, cosmids, and yeast artificial chromosomes; vectors are often recombinant molecules containing DNA sequences from several sources. A vector includes a viral vector: for example, adenovirus (icosahedral (20-sided) virus that  
15 contains dsDNA; there are over 40 different adenovirus varieties, some of which cause the common cold); adenoassociated viral vectors (AAV) which are derived from adenoassociated viruses and are smaller than adenoviruses; and retrovirus (any virus in the family Retroviridae that has RNA as its nucleic acid and uses the enzyme reverse transcriptase to copy its genome into the DNA of the host cell's chromosome;  
20 examples include VSV G and retroviruses that contain components of lentivirus including HIV type viruses).

### ***Polymers***

25 Polymers are used for drug delivery for a variety of therapeutic purposes. Polymers have also been used in research for the delivery of nucleic acids (polynucleotides and oligonucleotides) to cells with an eventual goal of providing therapeutic processes. Such processes have been termed gene therapy or anti-sense therapy. One of the several methods of nucleic acid delivery to the cells is the use of DNA-polycations  
30 complexes. It has been shown that cationic proteins like histones and protamines or synthetic polymers like polylysine, polyarginine, polyornithine, DEAE dextran, polybrene, and polyethylenimine may be effective intracellular delivery agents while small polycations like spermine are ineffective. The following are some important

principles involving the mechanism by which polycations facilitate uptake of DNA. Protein refers to a linear series of greater than 50 amino acid residues connected one to another as in a polypeptide.

- 5 Polycations provide attachment of DNA to the target cell surface. The polymer forms a cross-bridge between the polyanionic nucleic acids and the polyanionic surfaces of the cells. As a result the main mechanism of DNA translocation to the intracellular space might be non-specific adsorptive endocytosis which may be more effective than liquid endocytosis or receptor-mediated endocytosis. Furthermore, polycations are a convenient linker for attaching specific receptors to DNA and as result, DNA-  
10 polycation complexes can be targeted to specific cell types.

- Polycations protect DNA in complexes against nuclease degradation. This is important for both extra- and intracellular preservation of DNA. The endocytic step in  
15 the intracellular uptake of DNA-polycation complexes is suggested by results in which DNA expression is only obtained by incorporating a mild hypertonic lysis step (either glycerol or DMSO). Gene expression is also enabled or increased by preventing endosome acidification with  $\text{NH}_4\text{Cl}$  or chloroquine. Polyethylenimine, which facilitates gene expression without additional treatments, probably disrupts  
20 endosomal function itself. Disruption of endosomal function has also been accomplished by linking to the polycation endosomal-disruptive agents such as fusion peptides or adenoviruses.

- Polycations facilitate DNA condensation. The volume which one DNA molecule  
25 occupies in complex with polycations is drastically lower than the volume of a free DNA molecule.

A Lewis acid:Lewis base complex is an electron pair acceptor:electron pair donor.

- 30 A transition metal is a group of metallic elements in which the members have the filling of the outermost shell to eight electrons interrupted to bring the penultimate shell from 8 to 18 or 32 electrons and this includes elements 21-29 and 29-47 and 57-79 and all known elements from 89 on.

A chemical attachment is defined in this specification as a covalent, noncovalent bond or pi stacking between two atoms.

- 5 In this specification, a modifying chemical attachment is a chemical attachment, including a Lewis acid:Lewis base attachment but does not include electrostatic binding, hydrogen bonding, pi stacking, minor groove binding or intercalation.

### ***Routes of Administration***

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An intravascular route of administration enables a polymer or polynucleotide to be delivered to cells more evenly distributed and more efficiently expressed than direct injections. Intravascular herein means within a tubular structure called a vessel that is connected to a tissue or organ within the body. Within the cavity of the tubular structure, a bodily fluid flows to or from the body part. Examples of bodily fluid  
15 include blood, lymphatic fluid, or bile. Examples of vessels include arteries, arterioles, capillaries, venules, sinusoids, veins, lymphatics, and bile ducts. The intravascular route includes delivery through the blood vessels such as an artery or a vein (US patent application serial no. 08/975,573 is incorporated herein by reference).

20

An administration route involving the mucosal membranes is meant to include nasal, bronchial, inhalation into the lungs, or via the eyes.

### **Summary**

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### ***The Use of Chemically? Modified DNA for Nucleic Acid Delivery, Integration, and Gene Expression Utilizing Modifying chemical attachment Bonding Chemistry***

Efficient transport of polynucleotides into cells is vital for effective gene therapy.

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Since covalently modified nucleic acids can be expressed efficiently within cells, covalent modification of polynucleotides may be used to enhance cellular gene expression. Nucleic acids can also be modified at specific locations via modifying chemical attachment non-covalent attachment chemistries like cis-platinum. By

modifying polynucleotides (i.e. DNA, RNA or oligonucleotides) using modifying chemical attachment bonding chemistry, it is possible to attach a wide array of molecules (i.e. signals) that enhance uptake, expression or antisense activities of DNA, RNA or oligonucleotides in cells. Signals can be attached to polynucleotides that augment cell binding, cell internalization, endosome escape, cytoplasmic transport, nuclear localization, nuclear retention and/or gene expression (see signal section). Covalent modifications may also facilitate recombination of the delivered (modified) DNA with the endogenous unmodified DNA thus facilitating integration of the delivered DNA into the host chromosomes. Covalent attachment of polycations to nucleic acids can also be used to decrease the size of DNA-polycation complex.

In a preferred embodiment, polynucleotides (DNA or RNA or oligonucleotides) are covalently modified either within or outside of an expressible sequence to contain specific signals that enhance one or more of the following activities; cellular uptake, cytoplasmic transport, nuclear localization, gene expression, or chromosomal integration.

***The Use Of Modified DNA Utilizing Modifying chemical attachment Bonding Chemistry To Generate An Augmented Immune Response Against An Expressed Polypeptide.***

Gene based vaccines are currently being extensively studied as an attractive alternative to viral or protein based vaccines. For gene based vaccines to be useful, a robust immune response against the encoded "foreign" protein must be generated by the recipient organism. It is possible that chemical modification of DNA with any of a wide range of compounds or unusual conformations such as Z-DNA may induce immune response (K. Moelling, Gene Therapy, 5:573-574, 1998). The ability to chemically modify an expressible gene sequence with any of a wide range of signals or reactive species while maintaining its ability to be expressed into a polypeptide provides a new and powerful method for augmenting gene based immune responses.

***Cellular Delivery of Modified Polynucleotides Utilizing Modifying chemical attachment Bonding Chemistry***

Modified polynucleotides for use in gene therapy can be delivered to cells using the same methods used to deliver unmodified polynucleotides. These include delivery of naked polynucleotides through intravascular, intraperitoneal, intramuscular, oral, or  
5 direct intraparenchymal injections. Covalently modified polynucleotides for use in gene therapy can also be delivered to cells after complexing with cationic lipids, cationic polymers, cationic protein, transfection reagents, amphipathic polyamines, polyethylenimine and others. Covalently modified polynucleotides can also be delivered to cells after sequestration inside cationic, anionic or neutral liposomes,  
10 after inclusion within non-viral polyelectrolyte particles consisting of cationic and/or anionic polymers and/or cationic and/or polyanionic proteins. The covalently modified DNA is functional following delivery to cells in either a condensed state (see example 1) or in an uncondensed state (see examples 3 and 4).

15 Modified nucleic acids that retain the ability to be expressed have many uses. Modified nucleic acids can be targeted to specific cell types by attaching targeting ligands. Any ligand with specificity for a given cell or tissue type would be applicable. Some examples include, ligands targeting the asialoglycoprotein receptor of liver cells, cell type specific integrins on nearly any cell type, folate receptor on  
20 tumor cells, CD4 receptor on lymphoid cells, etc. The modifications can also result in increased expression by directing the nucleic acid to the nucleus where transcription takes place. This is accomplished by attaching nuclear locating peptides (NLS) to the nucleic acid. Escape of the nucleic acid from the endosome may be enhanced by attaching endosomal disrupting peptides or other compounds to the nucleic acid.  
25 Delivery to cells in vivo can be enhanced by the attachment of cationic or anionic charge to the nucleic acid resulting in a change in tertiary structure of the nucleic acid. A change in tertiary structure may lead to increased resistance to DNases, and an increased rate of egress from the bloodstream as a result of reduced radius of gyration (i.e. size). Furthermore, DNA compaction can be gained by attaching reactive groups  
30 such as sulfhydryls to the nucleic acid. The sulfhydryls can then be oxidized into disulfides resulting in a crosslinked compacted nucleic acid. Upon delivery to the cell cytoplasm these disulfides would be reduced by intracellular reduction pathways (both glutathione dependent and glutathione independent pathways) thereby returning

the nucleic acid to its native conformation. Any of the signals, ligands, peptides, or molecules attached to the nucleic acid can be attached to the nucleic acid in a reversible manner. This allows removal of the attached group at a desired location, for example a pH sensitive linkage between the nucleic acid reactive group and the signal/functional group could be used to release the attached group in areas of low pH such as the interior of endosomes or near tumors. Disulfide linkages could be used to release attached groups in the cytoplasm of cells. For example a multiply charged group such as di or tri-lysine could be attached to the *LabelIT*<sup>®</sup> molecule via a disulfide linkage. The nucleic acid would then be compacted during delivery of the nucleic acid to the cell. Once the nucleic acid escapes the endosome the disulfide linkages would be reduced, returning the nucleic acid to its native conformation.

### Brief Description of the Drawings

FIG. 1 is a print of an Agarose gel of DNA covalently modified with (4 +) *LabelIT*<sup>®</sup> Monomer.

FIG. 2 is a print of Atomic Force Microscopy (AFM) of DNA particles formed by the covalent attachment of *LabelIT*<sup>®</sup>-Trimer to plasmid DNA.

FIG. 3 is a graph illustrating fluorescence vs. time.

### Detailed Description

The present invention relates to a process of modifying a gene (either within or outside an expressible sequence) such that the gene can be efficiently expressed in a cell. The expressible gene sequence which is modified can be a part of circular piece of DNA such as a plasmid, or a linear piece of DNA. The expressible sequence can be modified with any of a wide range of compounds. Compounds can be attached to nucleic acids for a number of different reasons including: marking of the gene sequence for identification within cells, to augment its delivery into the cell over that of unmodified sequences, or to facilitate increased expression of the gene product. A compound can also be attached that would increase the frequency of stable integration of the modified DNA into the genomic DNA of an organism over that which is

obtained with unmodified DNA. Efficient stable integration of labeled DNA is highly desirable for creating stable cell transfectants in vitro, transgenic animals, transgenic plants or for gene therapy purposes. DNA can be efficiently modified covalently on the N7 position of guanine using alkylating agents such as nitrogen mustards, sulfur  
5 mustards, epoxides, aziridines, episulfides, dimethylsulfate, molecules containing activated cyclopropyl groups such as the CPI family of molecules, bromoacetamides, or non-covalently using cis-platinum based reagents. Expressible gene sequences modified on the N7 position of guanine can be efficiently expressed following introduction into cells.

10 In one preferred embodiment, compounds are attached to DNA at the N7 position and the expressible sequence remains functional upon introduction into a cell.

The present invention also relates to a process of delivering the modified gene to a cell. Delivering a gene means that the gene is placed in a position to become associated with the cell. The gene can be on the membrane of the cell or inside the  
15 cytoplasm, nucleus, or other organelle of the cell. The process of delivering a gene to a cell has also been commonly termed "transfection" or the process of "transfecting" and also it has been termed "transformation." The cell can be a mammalian cell that is within the tissue in situ. The cell can also have been removed and maintained in tissue culture in a primary, secondary, immortalized or transformed state.

20 The gene, as used in this specification, is a unit of coded information usually used to make a functional product. It is a polynucleotide and can be double-stranded DNA, single-stranded DNA, or a messenger RNA. The double-stranded DNA is typically derived from either plasmid DNA in bacteria or from polymerase chain reaction amplification (PCR). These polynucleotides contain a coding sequence for a  
25 polypeptide or protein and the associated sequences required for expression. For the DNA this includes a promoter, enhancer, 5' untranslated regions, 3' untranslated regions, introns, poly A addition site and transcription terminators. For RNA, a promoter, enhancer, poly A addition site, or transcription terminator would not be necessary. An oligonucleotide such as an antisense molecule that doesn't express a  
30 protein is excluded from this definition of a gene.

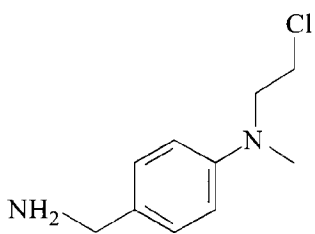
#### Attachment of A Signal Molecule to A Gene Without Preventing Its Expression



The gene transfer enhancing signal is attached utilizing modifying chemical attachment bonding chemistry such as a covalent bond to the gene using a variety of methods. They can be alkylating reagents or photoactivatable compounds. Examples of alkylating reagents include the use of mustards and the use of compounds

- 5 containing the CPI DNA alkylating moiety (cyclopropa-pyrrolo-indol) and its derivatives. All compounds in the CPI family include the functionality: 1,2,8,8a-tetrahydro-7-methylcyclopropa-[c]pyrrolo-[3,2-e]indol-4(5H)-one.

- Mustards are molecules consisting of a nucleophile and a leaving group separated by an ethylene bridge. After internal attack of the nucleophile on the  
10 carbon bearing the leaving group a strained three membered group is formed. This strained ring (in the case of nitrogen mustards an aziridine ring is formed) is very susceptible to nucleophilic attack. Thus allowing mustards to alkylate weak nucleophiles such as polynucleic acids. Mustards can have one of the ethylene bridged leaving groups attached to the nucleophile, these molecules are sometimes  
15 referred to as half-mustards; or they can have two of the ethylene bridged leaving groups attached to the nucleophile. these molecules can be referred to as bis-mustards. One class of mustards are R-chloride derivatives that contain the aromatic nitrogen mustard 4-[(2-chloroethyl)-methylamino]-benzylamine. We incorporate herein by reference a patent application entitled : A Method for Single-Step Attachment of a



- 20 Label to Target Molecules, Serial No. 09/982,485.  
R-chloride

A gene can be modified within the sequences required for expression.

- 25 Expression as used in this specification means the transcription of the gene into RNA or the translation of RNA into protein. DNA sequences necessary for expression or transcription include sequences such as promoters, enhancers, 5' untranslated regions,

3' untranslated regions, introns, poly A addition site and transcription terminators.

Modification within the expression cassette does not prevent expression of the gene.

In one preferred embodiment, this can be accomplished by first chemically modifying a piece of DNA which contains an expressible sequence such as an entire plasmid.

- 5 Thus the attachment can be to sequences either within or outside the sequences required for expression.

#### Gene Transfer Enhancing Signals

- 10 In a preferred embodiment, a chemical reaction can be used to attach a gene transfer enhancing signal to a nucleic acid. The gene transfer enhancing signal (or abbreviated as the Signal) is defined in this specification as a molecule that modifies the nucleic acid complex for more efficient delivery to a location (such as tissue) or location in a cell (such as the nucleus) either in culture or in a whole organism. By modifying the cellular or tissue location of the foreign gene, the expression of the
- 15 foreign gene can be enhanced.

- The gene transfer enhancing signal can be a protein, peptide, lipid, steroid, sugar, carbohydrate, (non-expressing) polynucleic acid or synthetic compound. The gene transfer enhancing signals enhance cellular binding to receptors, cytoplasmic transport to the nucleus and nuclear entry or release from endosomes or other
- 20 intracellular vesicles.

- Nuclear localizing signals enhance the targeting of the gene into proximity of the nucleus and/or its entry into the nucleus. Such nuclear transport signals can be a protein or a peptide such as the SV40 large T antigen NLS or the nucleoplasmin NLS. These nuclear localizing signals interact with a variety of nuclear transport factors
- 25 such as the NLS receptor (karyopherin alpha) which then interacts with karyopherin beta. The nuclear transport proteins themselves could also function as NLS's since they are targeted to the nuclear pore and nucleus. For example, karyopherin beta itself could target the DNA to the nuclear pore complex. Several peptides have been derived from the SV40 T antigen. These include a short NLS (H-
- 30 CGYGPKKKRKVGG-OH) or long NLS's (H-CKKKSSSDDEATADSQHSTPPKKRKVEDPKDFPSELLS-OH and H-CKKKWDDEATADSQHSTPPKKRKVEDPKDFPSELLS-OH). Other NLS peptides have been derived from M9 protein

(CYNDFGNYNNQSSNFGPMKQGNFGGRSSGPY), E1A (H-CKRGPKRPRP-OH), nucleoplasmin (H-CKKAVKRPAATKKAGQAKKKKL-OH), and c-myc (H-CKKKGPAAKRVKLD-OH).

Signals that enhance release from intracellular compartments (releasing  
5 signals) can cause DNA release from intracellular compartments such as endosomes (early and late), lysosomes, phagosomes, vesicle, endoplasmic reticulum, golgi apparatus, trans golgi network (TGN), and sarcoplasmic reticulum. Release includes movement out of an intracellular compartment into cytoplasm or into an organelle such as the nucleus. Releasing signals include chemicals such as chloroquine,  
10 bafilomycin or Brefeldin A1 and the ER-retaining signal (KDEL sequence), viral components such as influenza virus hemagglutinin subunit HA-2 peptides and other types of amphipathic peptides.

Cellular receptor signals are any signal that enhances the association of the gene with a cell. This can be accomplished by either increasing the binding of the  
15 gene to the cell surface and/or its association with an intracellular compartment, for example: ligands that enhance endocytosis by enhancing binding the cell surface. This includes agents that target to the asialoglycoprotein receptor by using asialoglycoproteins or galactose residues. Other proteins such as insulin, EGF, or transferrin can be used for targeting. Peptides that include the RGD sequence can be  
20 used to target many cells. Chemical groups that react with sulfhydryl or disulfide groups on cells can also be used to target many types of cells. Folate and other vitamins can also be used for targeting. Other targeting groups include molecules that interact with membranes such as lipids fatty acids, cholesterol, dansyl compounds, and amphotericin derivatives. In addition viral proteins could be used to bind cells.

25 In a preferred embodiment, a complex is formed by using a modifying chemical attachment for attaching a plurality of compound to a nucleic acid in an amount sufficient to change the tertiary structure of the nucleic acid. The change in tertiary structure allows for more efficient delivery of the complex to a cell, *in vivo*, when compared to compound attachment where the attachment is less strong such as  
30 ionic bonding.

In a preferred embodiment, a process for nucleic acid delivery includes preparing a nucleic acid molecule having an expressible sequence. A compound is attached to the nucleic acid molecule within the expressible sequence which allows

more than 40% expression of the expressible sequence as compared to the nucleic acid without compound attachment. The nucleic acid is delivered to a cell where the expressible sequence may be expressed.

In a preferred embodiment a signal is covalently attached to a plasmid (i.e. circular polynucleotide) randomly at a position either within or outside of the expressible sequence of the plasmid.

In another preferred embodiment the signal is attached to a polynucleotide either within or outside the expressible sequence and the signal is selected from a group of compounds that stimulate an enhanced immune reaction against the protein encoded by the expressible sequence.

In another preferred embodiment a polycation is covalently attached to a polynucleotide resulting in DNA compaction and negatively charged particle formation. The attachment of the polycation allows for the formation of DNA particles that have a different tertiary (3-dimensional) conformation than particles formed by non-covalent interactions. In one This indicates that charge neutralization is not required and particle formation is not the result of charge mediated DNA condensation.

#### **Example 1.**

DNA covalently modified (alkylated) with rhodamine is efficiently expressed in COS 7 cells following transfection.

#### **Methods.**

*DNA labeling* - Rhodamine molecules were covalently attached to plasmid DNA (pCILuc) encoding the reporter gene luciferase through an alkylation reaction. The plasmid, pCILuc was mixed with *LabelIT*<sup>®</sup> Rhodamine (Mirus Corporation, Madison WI) at three different ratios (1:0.2; 1:0.1; 1:0.05) (wt:wt) and incubated for 30 minutes at 37°C. Rhodamine-labeled DNA was purified and concentrated by ethanol precipitation. Rhodamine-labeling was confirmed by agarose gel electrophoresis in which a mobility shift of all rhodamine-labeled DNA was observed (data not shown). *Luciferase expression* - For gene expression studies rhodamine-labeled DNA was complexed with the transfection reagent TransIT LT-1 (Mirus Corporation) at a 1:3

(wt : vol) ratio and added to COS 7 cells according to manufacturer's recommendations. Transfected cells were grown at 37°C for 48 hours and harvested into luciferase buffer (0.1M KPO<sub>4</sub>, pH 7.8; 1mM DTT; 0.1% Triton X-100). Cell lysates were assayed for luciferase activity on a luminometer (Lumat LB 9507,  
 5 EG&G Berthold).

#### Results.

*LabelIT*<sup>®</sup> rhodamine facilitates the attachment of rhodamines primarily at guanine nucleotides so labeling occurs throughout the plasmid. The different levels of labeling  
 10 that was used results in the covalent attachment of about 1 rhodamine per 30 – 120 base pairs of DNA. Thus the protein coding sequence of the luciferase gene cassette would be expected to contain a large number of covalently modified guanine residues (~12 – 50 labels). To determine the effect of the covalent attachment of rhodamines on gene expression, luciferase activity of the modified plasmid DNA was compared to  
 15 the similarly transfected unmodified plasmid DNA. From these experiments we found that covalently modified pCILuc was expressed at levels similar to unmodified pCILuc.

<u>DNA Transfected</u>	<u>Luciferase Expression in liver</u> <u>(Relative to unmodified pCILuc)</u>
Naked DNA (pCILuc)	1.0
pCILuc-Rhodamine (0.2 : 1 labeling ratio, <i>LabelIT</i> <sup>®</sup> rhodamine: pDNA)	0.47
25 pCILuc-Rhodamine (0.1 : 1 labeling ratio, <i>LabelIT</i> <sup>®</sup> rhodamine: pDNA)	0.80
30 pCILuc-Rhodamine (0.05 : 1 labeling ratio, <i>LabelIT</i> <sup>®</sup> rhodamine: pDNA)	0.88

**Example 2.**

DNA chemically modified with DNP using a modifying chemical non-covalent attachment (cis-platinum) is expressed in COS7 cells following transfection as efficiently as unmodified DNA.

**Methods.**

*DNA labeling* – dinitrophenol (DNP) molecules were attached to plasmid DNA (pCILuc) encoding the reporter gene luciferase through a cis-platinum reaction. The plasmid, pCILuc was mixed with the labeling reagent (Versitag, NEN) at a 0.1:1 ratios (vol:wt) and incubated for 30 minutes at 85°C. DNP-labeled DNA was purified and concentrated by ethanol precipitation.

*Luciferase expression* - For gene expression studies DNP-labeled DNA was complexed with the transfection reagent TransIT LT-1 (Mirus Corporation) at a 1:3 (wt : vol) ratio and added to COS7 cells according to manufacturer's recommendations. Transfected cells were grown at 37°C for 48 hours and harvested into luciferase buffer (0.1M KPO<sub>4</sub>, pH 7.8; 1mM DTT; 0.1% Triton X-100). Cell lysates were assayed for luciferase activity on a luminometer (Lumat LB 9507, EG&G Berthold).

**Results.**

Versitag-DNP facilitates the attachment of DNP molecules primarily at guanine nucleotides so labeling occurs throughout the plasmid. To determine the effect of the non-covalent DNP attachment on gene expression, luciferase activity of the modified plasmid DNA was compared to the similarly transfected mock-labeled plasmid DNA. From these experiments we found that DNP modified pCILuc using a modifying chemical non-covalent attachment was expressed at about 50% that of unmodified pCILuc.

**DNA Transfected**
**Luciferase Expression COS7 cells**  
**(Relative to mock pCILuc)**

Mock labeled DNA (pCILuc)

1.0

pCILuc-DNP 0.49  
(0.1 : 1 labeling ratio, Versitag-DNP: pDNA)

### 5 **Example 3**

DNA covalently modified (alkylated) with digoxin is efficiently expressed in mouse liver hepatocytes following *in vivo* delivery.

#### Methods:

- 10 *DNA Labeling* - *LabelIT*<sup>®</sup> digoxin (Mirus Corporation, Madison WI) was used to covalently attach digoxin molecules to a plasmid DNA encoding the reporter gene luciferase (pCILuc). Three different labeling ratios were used to achieve varying amounts of plasmid labeling (0.1:1, 0.05:1, 0.025:1 wt:wt, labeling reagent to DNA). The digoxin labeled DNAs was purified and concentrated by ethanol precipitation.
- 15 Labeling was confirmed by agarose gel electrophoresis in which a mobility shift of all labeled DNA was observed (data not shown).

*In vivo gene delivery* – Digoxin-labeled and unlabeled pCILuc was delivered into mice via tail vein injections (Zhang et al., Human Gene Therapy, Vol. 10 (1999)). Briefly, labeled or unlabeled DNA in a physiologic salt solution was rapidly injected

20 (2.5 ml solution in ~7 seconds) into the tail vein of ~25g ICR mice (Harlan Sprague Dawley, Indianapolis, IN). Animals were sacrificed 1 day after injections and the livers surgically removed. Livers were homogenized in luciferase buffer (0.1M KPO<sub>4</sub>, pH 7.8; 1mM DTT; 0.1% Triton X-100) and assayed for luciferase activity on a luminometer (Lumat LB 9507, EG&G Berthold).

25

#### Results:

The luciferase encoding plasmid DNA, pCILuc, was covalently labeled with varying amounts of digoxin and compared to unmodified pCILuc in *in vivo* gene delivery assays. In concordance with the *in vitro* transfections, covalently modified pCILuc

30 expressed luciferase at least as efficiently as unmodified pCILuc.

#### DNA Injected

#### Luciferase Expression in liver (Relative to unmodified pCILuc)

	Naked DNA (pCILuc)	1.0
	pCILuc- digoxin (0.1 : 1 labeling ratio, <i>LabelIT</i> <sup>®</sup> digoxin : pDNA)	2.29
5	pCILuc- digoxin (0.05 : 1 labeling ratio, <i>LabelIT</i> <sup>®</sup> digoxin : pDNA)	4.67
10	pCILuc- digoxin (0.025 : 1 labeling ratio, <i>LabelIT</i> <sup>®</sup> digoxin : pDNA)	1.87

15

**Example 4:**

DNA covalently modified (alkylated) with biotin is efficiently expressed in mouse liver hepatocytes following in vivo delivery.

20 **Methods:**

*DNA Labeling* - *LabelIT*<sup>®</sup> biotin (Mirus Corporation, Madison WI) was used to covalently attach biotin molecules to a plasmid DNA encoding the reporter gene luciferase (pCILuc). Four different labeling ratios were used to achieve varying amounts of plasmid labeling. The biotin labeled DNAs was purified and concentrated  
25 by ethanol precipitation. Labeling was confirmed by agarose gel electrophoresis in which a mobility shift of all labeled DNA was observed (data not shown).

*In vivo gene delivery* - Biotin-labeled and unlabeled pCILuc was delivered into mice via tail vein injections (Zhang et al., Human Gene Therapy, Vol. 10 (1999)). Briefly, labeled or unlabeled DNA in a physiologic salt solution was rapidly injected (2.5 ml  
30 solution in ~7 seconds) into the tail vein of ~25g ICR mice (Harlan Sprague Dawley, Indianapolis, IN). Animals were sacrificed 1 day after injections and the livers surgically removed. Livers were homogenized in luciferase buffer (0.1M KPO<sub>4</sub>, pH



7.8; 1mM DTT; 0.1% Triton X-100) and assayed for luciferase activity on a luminometer (Lumat LB 9507, EG&G Berthold).

Results:

- 5 The luciferase encoding plasmid DNA, pCILuc, was covalently labeled with varying amounts of biotin and compared to unmodified pCILuc in *in vivo* gene expression assays. Following intravascular delivery (tail vein injection) to liver hepatocytes, biotinylated pCILuc was expressed at least as efficiently as unmodified pCILuc.

10 <u>DNA Injected</u>	<u>Luciferase Expression in liver</u> (Relative to unmodified pCILuc)
Naked DNA (pCILuc)	1.0
pCILuc-biotin 15 (0.2 : 1 labeling ratio, <i>LabelIT</i> <sup>®</sup> biotin : pDNA)	0.87
pCILuc-biotin (0.1 : 1 labeling ratio, <i>LabelIT</i> <sup>®</sup> biotin: pDNA)	3.25
20 pCILuc- biotin (0.05 : 1 labeling ratio, <i>LabelIT</i> <sup>®</sup> biotin: pDNA)	5.39
pCILuc- biotin (0.025 : 1 labeling ratio, <i>LabelIT</i> <sup>®</sup> biotin: pDNA)	1.41
25	

### Example 5

- 30 The covalent attachment of a peptide signal (nuclear localizing signal) to an expressible sequence enhances gene expression *in vivo*.

Methods:

#### DNA Labeling.

A Nuclear Localizing Sequence peptide (NLS, CPKKKRKVEDG) derived from the SV40 large T antigen and a control peptide were covalently attached to a DNA reactive compound (*LabelIT*<sup>®</sup>-Amine; Mirus Corporation). *LabelIT*<sup>®</sup> is a nitrogen mustard derivative that alkylates nucleic acid, thus forming a covalent bond between the reagent and the nucleic acid. After attachment of peptides to *LabelIT*<sup>®</sup>, the *LabelIT*<sup>®</sup>-NLS and the *LabelIT*<sup>®</sup>-control peptide were reacted with plasmid DNA (pCILuc) at the ratios indicated below. The DNA alkylation reaction facilitated by the *LabelIT*<sup>®</sup> compounds results in covalent attachment of the peptides directly to the DNA. In this alkylation reaction the peptides are covalently attached to sequences throughout the plasmid both inside the expressible gene sequence and outside.

#### Intravascular Injections.

Ten micrograms of covalently modified or control DNA was injected into the tail vein of ICR mice (Harlan Sprague Dawley) as previously described (Zhang et al. Human Gene Therapy, 10:1735-1737). Twenty four hours after injection, livers were excised and cell extracts were prepared and assayed for reporter gene activity (luciferase).

#### Results;

Plasmid DNA encoding the luciferase gene (pCILuc) was covalently modified via the attachment of a peptide signal (nuclear localizing sequence; CPKKKRKVEDG) or a control peptide (Mirus 017; IAEYIPLETDLG) and injected into the tail vein of mice using an in vivo gene delivery method. All covalently modified plasmid constructs were compared to unmodified pCILuc for gene expression capabilities. Reporter gene expression (luciferase) was assayed in the liver after 24 hours. Both sets of animals that received DNA with covalently attached NLS peptides displayed levels of gene expression higher than both naked DNA controls and DNA modified with a control (non-NLS peptide). Two conclusions can be drawn from these results. 1) The covalent attachment of peptides to expressible sequences does not inhibit gene expression as compared to unmodified plasmid DNA; and 2) the attachment of a functionally active peptide (i.e. NLS) augments gene expression.

	<u>DNA Injected</u>	<u>Luciferase Expression in liver (nanograms)</u>
5	Naked DNA (pCILuc)	2330
	pCILuc-NLS (0.2 : 1 labeling ratio, peptide : pDNA)	3720
10	pCILuc-NLS (0.1 : 1 labeling ratio, peptide : pDNA)	3510
	pCILuc-control peptide (0.2 : 1 labeling ratio, peptide : pDNA)	1440
15	pCILuc-control peptide (0.1 : 1 labeling ratio, peptide : pDNA)	1500

### Example 6

20

In vivo gene delivery of a covalently modified gene sequence results in an enhanced immune response against the plasmid encoded expressible sequence.

### Methods:

25

*DNA labeling* - Rhodamine molecules were covalently attached to plasmid DNA (pCILuc) encoding the reporter gene luciferase through an alkylation reaction. The plasmid, pCILuc was mixed with *LabelIT*<sup>®</sup> Rhodamine (Mirus Corporation, Madison WI) at two different ratios (1:0.2; 1:0.1) (wt:wt) and incubated for 30 minutes at 37°C. Rhodamine-labeled DNA was purified and concentrated by ethanol precipitation. Rhodamine-labeling was confirmed by agarose gel electrophoresis in which a mobility shift of all rhodamine-labeled DNA was observed (data not shown). Rhodamine-labeled and unlabeled pCILuc was delivered into mice via tail vein

injections (Zhang et al., Human Gene Therapy, Vol. 10 (1999)). Briefly, labeled or unlabeled DNA in a physiologic salt solution was rapidly injected (2.5 ml solution in ~7 seconds) into the tail vein of ~25g ICR mice (Harlan Sprague Dawley, Indianapolis, IN). Three weeks after injection blood was obtained from each of the injected animals and tested for anti-luciferase antibody production on an ELISA assay.

ELISA assay - Plasma samples were obtained through retro-orbital bleeding at several time points following pDNA delivery. The presence of anti-luciferase antibodies was determined by an indirect ELISA. Plates were coated with luciferase protein (Promega, Madison, WI). Serial dilutions of mouse serum were assayed and anti-luciferase antibodies were detected by HRP-conjugated anti-mouse IgG (Sigma, St. Louis, MO), followed by TMB color development (Sigma, St. Louis, MO).

#### Results:

To determine if covalent attachment of a compound (rhodamine) to an expressible sequence could augment an antibody response against the encoded antigen, plasmid DNA expressing the luciferase gene (pCILuc) was covalently modified with rhodamine at two different levels of modification and injected into the tail vein of a mouse (see Zhang et al., Human Gene Therapy, Vol. 10 (1999)). Genes delivered using this methodology are expressed efficiently in liver hepatocytes and a number of other organs. Blood was collected at 3 weeks post-injection and serum was assayed for anti-luciferase antibodies.

25

<u>Injected DNA</u>	<u>ELISA Reading (450 nm)</u>
	<u>Serum dilution (1:100)</u>
Background Control	0.0318
Unlabeled DNA	0.085 (n=6)
Rhodamine-labeled DNA	0.1565 (n=4)

30

Conclusion:

As expected, injections of plasmid DNA encoding the luciferase gene resulted in antibody production against the luciferase protein by 3 weeks post-injection. However, the covalent attachment of a compound (rhodamine) at positions both  
5 within and outside of the expressible sequence facilitated increased antibody production against the “foreign” antigen (i.e. luciferase) as indicated by the increased ELISA values.

#### 10 **Example 7.**

Formation of novel DNA containing particles following the covalent attachment of polycations to polynucleotides under non-condensing conditions.

#### 15 **Methods:**

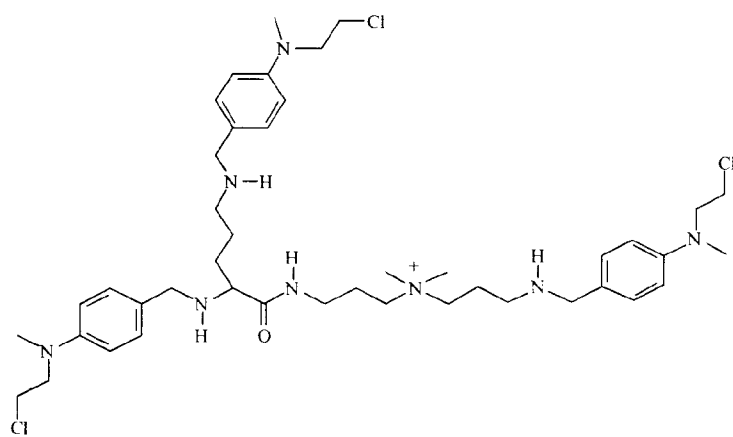
*Synthesis:* *Label IT*<sup>®</sup> Trimer was prepared by adding 4-[(2-chloroethyl)-methylamino]-benzaldehyde was added to the  $\alpha$  and  $\epsilon$  amino groups of the lysine subunit of (4 +) *Label IT*<sup>®</sup> via reductive amination. The final product was purified by  
20 HPLC.

(4 +) *Label IT*<sup>®</sup> was prepared by reacting the amine terminus of Mirus' commercially available *Label IT*<sup>®</sup> - Amine and  $N\alpha, N\epsilon$ -di-*t*-BOC-L-Lysine N-hydroxysuccinimide ester (Sigma Chemical Co.). The final product is generated by removal of the BOC  
25 protecting groups with trifluoroacetic acid. The final product was purified by HPLC.

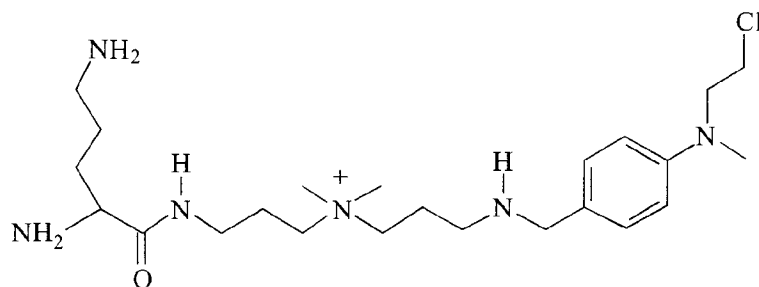
Rhodamine (4 +) *Label IT*<sup>®</sup> was prepared by coupling the amine terminus of Mirus' commercially available *Label IT*<sup>®</sup> - Amine with Fmoc-Lysine (BOC)OH (NovaBiochem) using carbodiimide chemistry. The BOC group was removed from  
30 the  $\epsilon$  amino group and the resulting amine was reacted with  $N\alpha, N\epsilon$ -di-*t*-BOC-L-Lysine N-hydroxysuccinimide ester (Sigma Chemical Co.). Following removal of the Fmoc group TAMRA-X, SE (Molecular Probes) was added to the  $\alpha$  amino group.

The final product was generated by removing the remaining BOC groups with trifluoroacetic acid, followed by HPLC purification.

DNA labeling with (4 +) *LabeIT*<sup>®</sup> Monomer. 105 micrograms of plasmid DNA (pCILuc) was labeled with 400 micrograms of the (4 +) La *LabeIT*<sup>®</sup> -monomer for increasing amounts of time (0, 1, 20, 40, 70, 110, 160 and 240 minutes). The labeling reaction was performed in the presence of 0.5 M NaCl which serves to inhibit the condensation of DNA by the unattached 4+ charged cation. Thus only DNA that has been covalently modified with the reactive *LabeIT*<sup>®</sup> will demonstrate retardation upon agarose gel electrophoresis. All samples labeled were electrophoresed on a 0.8% agarose gel and visualized by ethidium bromide staining.

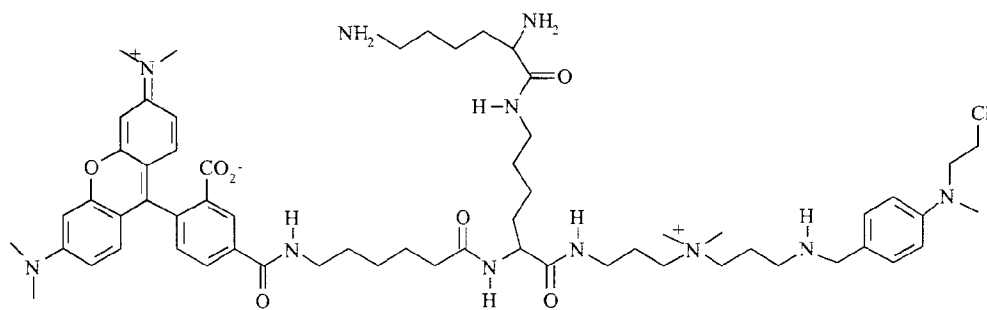


**LabelIT® Trimer (4 positive charges)**



5

**(4 +) LabelIT® Monomer (4 positive charges)**



Rhodamine (4 +) *Label IT*

**Results:**

The covalent attachment of cationic compounds containing 4 positive charges results in a DNA conformational change. The level of conformational change increases as a function of time. By 240 minutes all the plasmid DNA has a dramatically altered conformation in which the majority of the DNA is retarded in the gel (see FIG. 1) during electrophoresis. Without the stable attachment of the oligocations to the DNA (in the presence of 0.5 M NaCl), no DNA conformational change is observed.

**Example 8.**

DNA particles formed by the covalent attachment of polycations using the *LabelIT*<sup>®</sup>Trimer facilitate efficient gene expression in vivo.

**Methods:**

*DNA Labeling* - *LabelIT*<sup>®</sup>Trimer was used to covalently modify plasmid DNA encoding the reporter gene luciferase (pCILuc). Plasmid DNA was mixed with 3 different concentrations of *LabelIT*<sup>®</sup>Trimer (40, 90, and 200 ug) and allowed to incubate for 2 hours at 20°C and 12 hours at 4°C. Labeling was confirmed by agarose gel electrophoresis in which a mobility shift of all labeled DNA was observed.

*Atomic Force Microscopy* – Particles prepared using 175 micrograms of pDNA and 90 micrograms of *LabelIT*<sup>®</sup>Trimer were diluted to 1 mg/ml DNA, dried onto grids, and analyzed for size and shape using a Digital Instruments Nanoscope Scanning Probe Microscope.

*In vivo gene delivery* – Fifty micrograms of trimer-labeled and unlabeled pCILuc was delivered into mice via tail vein injections (Zhang et al., Human Gene Therapy, Vol. 10 (1999)). Briefly, labeled or unlabeled DNA in a physiologic salt solution was rapidly injected (2.5 ml solution in ~7 seconds) into the tail vein of ~25g ICR mice (Harlan Sprague Dawley, Indianapolis, IN). Animals were sacrificed 1 day after injections and the livers surgically removed. Livers were homogenized in luciferase



buffer (0.1M KPO<sub>4</sub>, pH 7.8; 1mM DTT; 0.1% Triton X-100) and assayed for luciferase activity on a luminometer (Lumat LB 9507, EG&G Berthold).

#### Results:

5

Plasmid DNA encoding the luciferase gene (pCILuc) was covalently modified via the attachment of a trifunctional DNA reactive polycation. Attachment of this trimer facilitates a conformational change in the structure of the DNA as observed in agarose gel electrophoresis. To determine the exact conformation the covalently modified

10 DNA was adopting, atomic force microscopy was used to visualize the particles (see AFM below). From this analysis it was found that the covalent attachment of oligocations of 4+ charges resulted in a range of stable spheroid particles of between approximately 20 and 350 nanometers in diameter. To determine if the particles remained functional, all covalently modified plasmid constructs were compared to

15 unmodified pCILuc for gene expression capabilities following in vivo gene delivery into the tail vein of mice. Reporter gene expression (luciferase) was assayed in the various organs after 24 hours.

20 **See FIG. 2 for Atomic Force Microscopy (AFM) of DNA particles formed by the covalent attachment of *LabelIT*<sup>®</sup>-Trimer to plasmid DNA.**

#### **Gene Expression of pDNA Covalently Modified with *LabelIT*<sup>®</sup>-Trimer**

<u>DNA Injected</u>	<u>Luciferase Expression</u>
	(Relative to unmodified pCILuc)
Unmodified DNA (pCILuc)	
Liver	1.0
Spleen	1.0
30 Kidney	1.0
Heart	1.0
Lung	1.0

pCILuc- *LabelIT*<sup>®</sup> Trimer  
(40 ug Trimer : 175 ug pDNA)

	Liver	1..81
5	Spleen	2.05
	Kidney	0.72
	Heart	5.30
	Lung	2.79

10 pCILuc- *LabelIT*<sup>®</sup> Trimer  
(90 ug Trimer : 175 ug pDNA)

	Liver	0.69
	Spleen	1.83
15	Kidney	0.08
	Heart	1.47
	Lung	0.87

20 pCILuc- *LabelIT*<sup>®</sup> Trimer  
(200 ug Trimer : 175 ug pDNA)

	Liver	0.07
	Spleen	1.14
	Kidney	0.02
25	Heart	1.78
	Lung	0.47

#### Conclusions:

In all organs, at the 40μg level, modified DNA displayed levels of expression  
comparable to or greater than unmodified DNA. Even at the highest levels of  
modification, gene expression in the heart and spleen was greater than the unmodified  
controls. These results indicate that covalent “crosslinking” of a DNA molecule does  
not inhibit expression and that the change in conformation resulting from this covalent

attachment (both within and outside of the expressible sequences) may be beneficial for in vivo gene delivery.

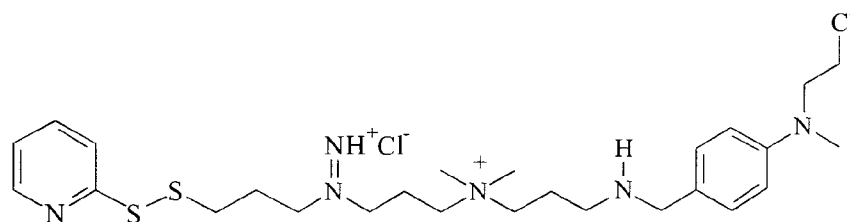
### Example 9.

5

Compaction of nucleic acids by disulfide exchange of covalently attached sulfhydryl groups.

Methods:

- 10 Pyridyldithio-*Label IT*<sup>®</sup> was prepared by reacting the amine terminus of Mirus' commercially available *Label IT*<sup>®</sup>-Amine with Traut's reagent (Pierce Chemical Co.). The sulfhydryl group that is generated by this reaction was trapped and protected as a pyridyldithio group with Aldrithiol<sup>®</sup>-2 (Aldrich Chemical Co.). The final product was purified via HPLC.
- 15 Plasmid DNA (pCI Luc) was modified at a 0.2:1 wt:wt (*Label IT*<sup>®</sup>:DNA) ratio using Pyridyldithio-*Label IT*<sup>®</sup> (see below). Following labeling the DNA was incubated at 4 °C overnight. The incubation period allows disulfide exchange to take place resulting in cross linked DNA. The modified sample was analyzed by agarose gel electrophoresis.



20 Pyridyldithio-*Label IT*<sup>®</sup>

Results:

Following overnight disulfide exchange, the pyridyldithio-*Label IT*<sup>®</sup> modified DNA remained in the well while control DNA labeled with bromoacetamide -*Label IT*<sup>®</sup>

- 25 entered the gel in a manner typical of *Label IT*<sup>®</sup> modified DNA. (i.e. the modified DNA enters the gel but shows retardation due to charge neutralization caused by the labeling process). This indicates that the pyridyldithio cross-linked DNA formed particles that could not enter the gel (similar to particles formed by crosslinking with

the *LabelIT*<sup>®</sup> Trimer; see example 7). When DNA was modified with the non-crosslinkable bromoacetamide groups, no particle formation occurs and the DNA migrates into the agarose gel.

5   **Example 10.**

Compaction of nucleic acid by oxidation of covalently attached sulfhydryl groups

Plasmid DNA (pCI Luc) was modified at a 0.2:1 wt:wt (*Label IT*<sup>®</sup>:DNA) ratio using  
10   pyridyldithio-*Label IT*<sup>®</sup> (see example 9). The pyridyldithio protecting groups were removed by reduction with TCEP (Pierce Chemical Co.). The nucleic acid was compacted by oxidizing the resulting sulfhydryl groups into disulfide bonds forming intramolecular crosslinks in the plasmid.

15   **Results:**

As was observed with disulfide exchange reaction (see example 9), disulfide bond formation facilitated intramolecular crosslinking which resulted in particle formation (complexes did not enter the well during electrophoresis). When the same disulfide crosslinked particles were reduced with DTT, DNA migrated into the gel.

20

**Example 11.**

Covalent attachment of an oligocation (4+ charges) facilitates DNA conformational changes under high salt conditions.

25

**Methods:**

Two micrograms of fluorescein labeled DNA (FI-DNA) was incubated with 20 micrograms of *LabelIT*<sup>®</sup>-Trimer (see example 8) in the presence of increasing amounts of unlabeled DNA (5.9, 12.1, 24.4, 49.1, 92.4 micromolar) in 1 ml of buffer  
30   consisting of 10mM HEPES, 500mM NaCl.

Fluorescence intensity (i.e. fluorescence quenching) of the fluorescein labeled DNA was monitored as a function of time on a Shimadzu fluorescence spectrophotometer (Em 518 nanometers).

#### Results:

When DNA labeled with fluorescein (or other fluorophores) undergoes conformational change in the form of compaction or condensation, the total emitted  
5 fluorescence decreases as a result of fluorescent quenching. This occurs when the fluorophores come in close proximity with each other as happens during DNA collapse. During polycation mediated particle formation, DNA condensation (and hence fluorescent quenching of fluorescein labeled DNA) occurs when the positive to negative charge ratio is  $> 0.9$ . At ratios of positive to negative charge  $< 0.9$ , DNA  
10 condensation does not occur. To determine if the covalent attachment of oligocations (4+ charge) facilitated fluorescence condensation, fluorescein labeled pDNA was mixed with *LabelIT*<sup>®</sup>-Trimer in the presence of increasing amounts of unlabeled pDNA in the presence of 500 mM salt (NaCl). At this concentration of salt, even an excess of the oligocation with 4+ charges will not condense pDNA. However,  
15 following the covalent attachment of the *LabelIT*<sup>®</sup>-Trimer oligocation to the plasmid DNA, fluorescent quenching of the labeled pDNA (i.e. DNA condensation) occurs at all ratios of positive to negative charges (see FIG. 3).

#### Conclusions:

20 Covalent attachment of *LabelIT*<sup>®</sup> Trimer (4+ charges) facilitates fluorescent quenching (i.e. DNA compaction) under a wide range of different oligocation to DNA ratios. The fluorescence quenching assay was performed in the presence of 0.5 M NaCl which would prevent non-covalent oligocation mediated DNA condensation at these charge ratios. This indicates that the covalent attachment of the oligocation  
25 facilitates the formation of a novel condensed-DNA containing particle and supports the observations of the other examples.

The foregoing is considered as illustrative only of the principles of the invention. Further, since numerous modifications and changes will readily occur to those skilled in the art, it is not desired to limit the invention to the exact construction  
30 and operation shown and described. Therefore, all suitable modifications and equivalents fall within the scope of the invention.

**We Claim:**

1. A process for nucleic acid delivery to a cell, comprising:
  - a) preparing a nucleic acid molecule having an expressible sequence;
  - b) attaching a compound to the nucleic acid molecule within the expressible sequence, utilizing a modifying chemical attachment; and,
  - c) delivering the nucleic acid to a cell where the expressible sequence is expressed.
2. The process of claim 1 wherein the level of expression obtained is greater than 40% of the level of expression obtained from the expressible sequence not having a modifying chemical attachment.
3. The process of claim 2 wherein the compound comprises a nucleic acid transfer enhancing signal.
4. The process of claim 3 wherein the nucleic acid transfer enhancing signal is selected from the group consisting of a nuclear localizing signal, a ligand that binds a receptor, and a releasing signal.
5. A process of claim 1 wherein the compound is selected from a group of consisting of an enhanced immune response molecule, an antigen, an antibody, a hapten, a membrane active compound, a peptide, a polymer, a polyion, and a fluorescent compound.
6. A process of claim 1 whereby the compound is attached to the N7 position of guanine.
7. The process of claim 1 wherein step of attaching comprises modifying the nucleic acid using an alkylating molecule.
8. The process of claim 7 wherein the alkylating molecule is selected from the group consisting of a mustard and a 3-membered ring system.

9. The process of claim 8 wherein the mustard is selected from the group consisting of a nitrogen mustard and a sulfur mustard.
10. The process of claim 9 wherein the 3-membered ring system is selected from the group consisting of aziridines, oxiranes, cyclopropyls, and episulfides.
11. The process of claim 9 wherein the nitrogen mustard consists of an R-chloride derivative.
12. The process of claim 7 wherein the 3-membered ring system consists of a CPI moiety.
13. The process of claim 1 wherein the nucleic acid consists of double-stranded and single stranded DNA.
14. A process of claim 1 wherein the step of attaching the compound comprises forming a Lewis acid:Lewis base complex, wherein the Lewis acid is not hydrogen.
15. A process of claim 14 wherein the Lewis acid is a transition metal.
16. A process of claim 15 wherein the Lewis acid is platinum.
17. A process of claim 14 wherein the Lewis base is N7 of guanine.
18. A complex for delivering nucleic acid to a cell, comprising: a plurality of a compound attached by a modifying chemical attachment to the nucleic acid in an amount sufficient to alter the tertiary structure of the nucleic acid when compared to the complex wherein the compound is not attached.

19. A complex of claim 18 wherein the change in tertiary structure results in a compaction or reduction in volume or a reduction in hydrodynamic radius of the nucleic acid.
20. A complex of claim 18 wherein the change in tertiary structure results in a retention of greater than 40% expression of the expressible sequence when compared to unmodified nucleic acid.
21. A complex of claim 18 wherein the compound attached to the nucleic acid is net positive, neutral, or net negative charge.
22. A complex of claim 18 whereby the compound is attached to the N7 position of guanine.
23. The complex of claim 18 wherein step of attaching comprises modifying the nucleic acid using an alkylating molecule.
24. The complex of claim 23 wherein the alkylating molecule is selected from the group consisting of a mustard and a 3-membered ring system.
25. The complex of claim 24 wherein the mustard is selected from the group consisting of a nitrogen mustard and a sulfur mustard.
26. The complex of claim 25 wherein the 3-membered ring system is selected from the group consisting of aziridines, oxiranes, cyclopropyls, and episulfides.
27. The complex of claim 26 wherein the nitrogen mustard consists of an R-chloride derivative.
28. The complex of claim 24 wherein the 3-membered ring system consists of a CPI moiety.



29. A complex of claim 18 wherein the step of attaching the compound comprises forming a Lewis acid:Lewis base complex, wherein the Lewis acid is not hydrogen.
30. A complex of claim 29 wherein the Lewis acid is a transition metal.
31. A complex of claim 30 wherein the Lewis acid is platinum.
32. A complex of claim 29 wherein the Lewis base is N7 of guanine.
33. An *in vivo* cell delivery complex, comprising: a complex formed by the process of claim 1.

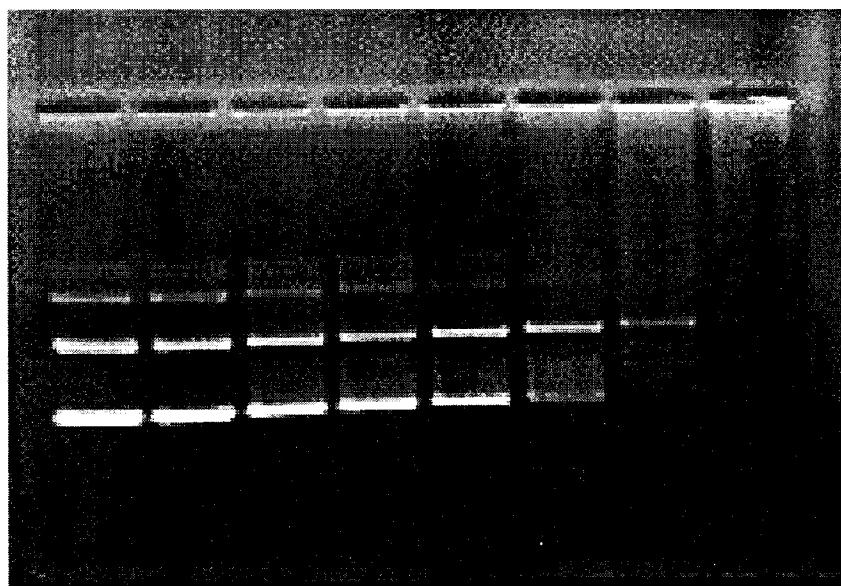


FIG. 1

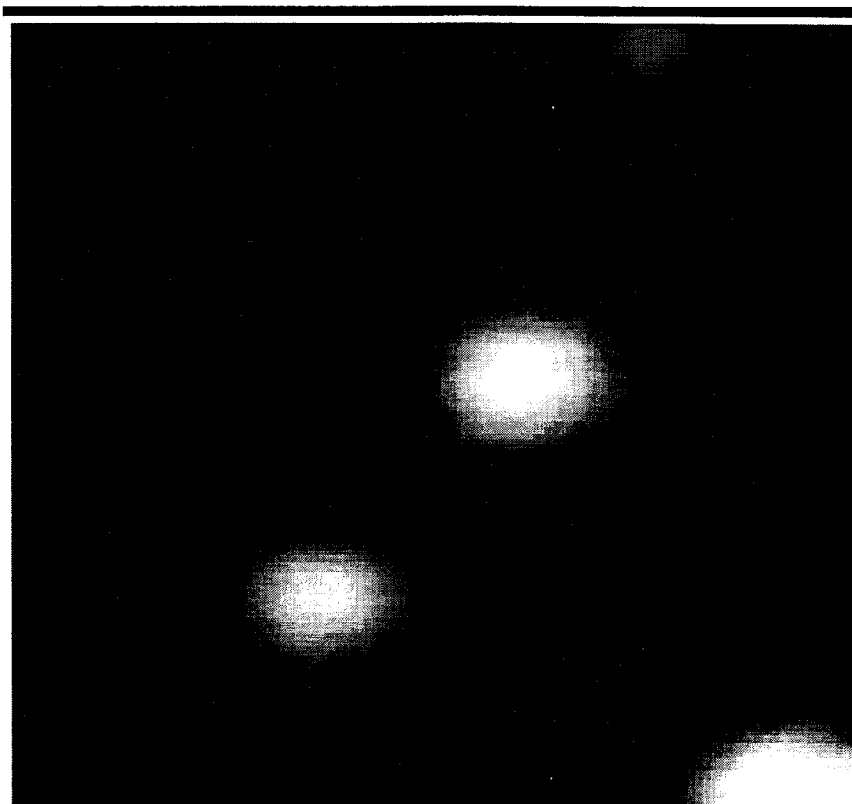


FIG. 2

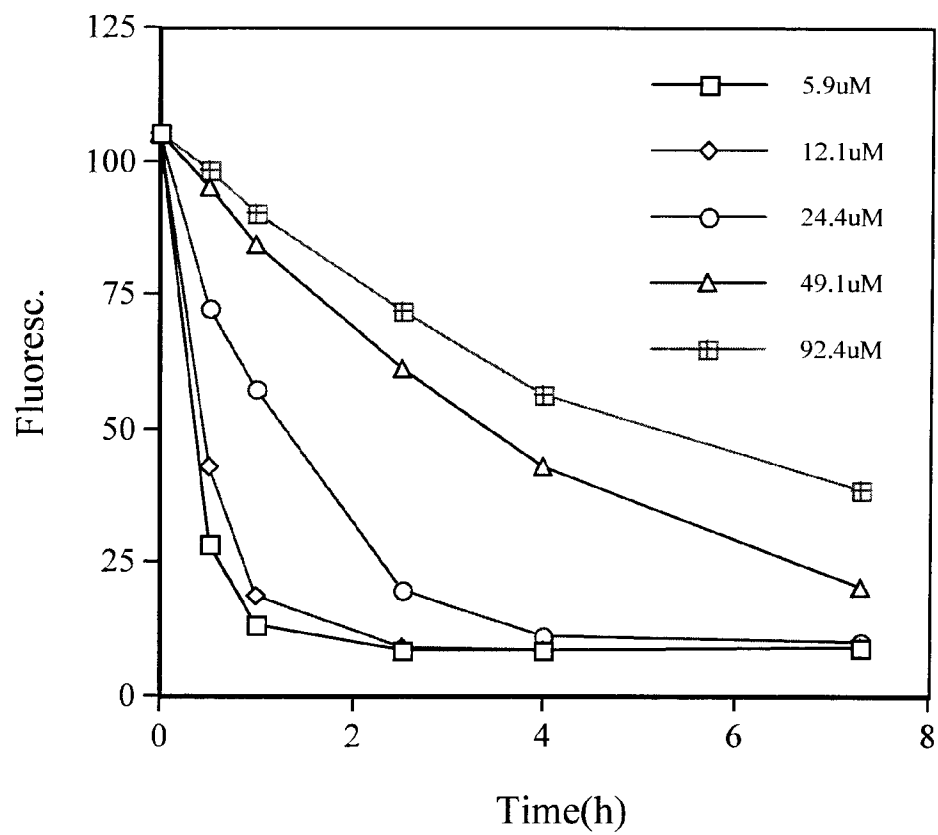


FIG. 3

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US00/21067

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : A61K 48/00; C12N 15/00, 15/63

US CL : 514/44; 435/320.1, 325, 455

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/44; 435/320.1, 325, 455

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  
NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAS ONLINE, MEDLINE

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LEAHY ET AL. Novel biotinylated Plasmid Expression Vectprs Retain Biological Function and Can Bind Strptavidin, BioConjugate Chem. 1996, Vol. 7, pages 545-551, especially page 548.	1-5, and 33
Y	DEAN. Import of Plasmid DNA into the Nucleus Is Sequence Specific. Experimental Cell Research. 1997, Vol. 230, pages 293-302, especially page 293.	1-33
Y	US 5,241,060 A (ENGELHARDT et al) 31 August 1993, columns 2 and 3, especially column 4.	1-33
A	US 6,093,701A (WOLFF et al) 25 July 2000, columns 5-20, especially columns 6-12.	1-33

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	* & * document member of the same patent family
"C" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

19 SEPTEMBER 2000

Date of mailing of the international search report

12 OCT 2000

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